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# A Concentration Addition Model to Assess Activation of the Pregnane X Receptor (PXR) by Pesticide Mixtures Found in the French Diet

Georges de Sousa*,[1], Ahmad Nawaz*,[2], Jean-Pierre Cravedi†, and Roger Rahmani*

*UMR 1331 TOXALIM (Research Centre in Food Toxicology), French National Institute for Agricultural Research (INRA), Laboratory of Xenobiotic's Cellular and Molecular Toxicology, 400 route des Chappes, BP 167, Sophia-Antipolis Cedex, France and †UMR 1331 TOXALIM (Research Centre in Food Toxicology), French National Institute for Agricultural Research (INRA), 180 chemin de Tournefeuille—BP 93173, Toulouse, Cedex 3, France

[1]To whom correspondence should be addressed at Laboratory of Xenobiotic's Cellular and Molecular Toxicology, 400 route des Chappes, BP 167, Sophia-Antipolis Cedex, France. Fax: +33-4-92-38-6401 E-mail: desousa@sophia.inra.fr or rahmani@sophia.inra.fr.

[2] Present address: Integrated Genomic, Cellular and Biotechnology Laboratory, Department of Entomology, University of Agriculture, Faisalabad. (UAF), Pakistan.

## ABSTRACT

French consumers are exposed to mixtures of pesticide residues in part through food consumption. As a xenosensor, the pregnane X receptor (hPXR) is activated by numerous pesticides, the combined effect of which is currently unknown. We examined the activation of hPXR by seven pesticide mixtures most likely found in the French diet and their individual components. The mixture's effect was estimated using the concentration addition (CA) model. PXR transactivation was measured by monitoring luciferase activity in hPXR/HepG2 cells and CYP3A4 expression in human hepatocytes. The three mixtures with the highest potency were evaluated using the CA model, at equimolar concentrations and at their relative proportion in the diet. The seven mixtures significantly activated hPXR and induced the expression of CYP3A4 in human hepatocytes. Of the 14 pesticides which constitute the three most active mixtures, four were found to be strong hPXR agonists, four medium, and six weak. Depending on the mixture and pesticide proportions, additive, greater than additive or less than additive effects between compounds were demonstrated. Predictions of the combined effects were obtained with both real-life and equimolar proportions at low concentrations. Pesticides act mostly additively to activate hPXR, when present in a mixture. Modulation of hPXR activation and its target genes induction may represent a risk factor contributing to exacerbate the physiological response of the hPXR signaling pathways and to explain some adverse effects in humans.

**Key words:** hPXR; pesticides; exposure; concentration addition; mixture

Pesticides constitute a major challenge in food and environmental safety. France is the highest consumer of pesticides in Europe and overall third in the world, with ~80,000 tons used per year. Although pesticides are undeniably useful for society, most of them pose some risk of harm to humans by virtue of their design to kill or adversely affect living organisms (Relyea, 2009). Considering the multiple sources of human exposure to these substances (i.e., through food, water, home, work, etc.), their potentials hazards must be taken seriously, as highlighted by EFSA & INSERM reports (Pesticides and human health, 2013). Pesticides represent a ubiquitous component of our environment, with many known to act as endocrine-disrupting chemicals (EDCs). By interfering with nuclear hormone receptors (NRs), they can produce developmental, reproductive, neurological,

immune, metabolic diseases, and cancer (Diamanti-Kandarakis *et al.*, 2009).

Moreover and above all, due to the broad spectrum of pesticide uses, consumers are exposed to mixtures of residues, the combined effects of which are largely underdocumented. Indeed, the estimation of health risks caused by pesticides remains largely based on Toxicological Reference values (acceptable daily intake, hazards, and risk index) for the substances taken individually. The European Food Safety Agency has acknowledged that an ~70,000 food samples analyzed in 2008, 47% contained detectable residues, 26.7% contained at least two pesticides, one-third of which contained over four. These pesticides in mixtures could, therefore, interact and unexpectedly impact human health, via additive, greater- or less than additive effects. Specific experimental approaches are essential to address these questions because of the large number of pesticides added and their effects in combination, which make mixture toxicological assessment impossible using classical methods. Both *in vitro* and theoretical approaches are required, that take into account similar, different or mixed modes of action, i.e., human cell culture coupled with concentration addition (CA) or independent action modeling. This approach could ultimately allow the prediction of the effects of mixtures from knowledge available on the effects of single chemicals.

Our Pericles research program aimed firstly to define the main pesticide mixtures to which the French general population is most heavily exposed to via their diet (Crepet *et al.*, 2013). The individual food consumption data for these pesticides were obtained from the French national consumption survey (the individual and national study on food consumption; INCA2) programs. On the whole, seven mixtures consisting of two to six pesticides were extracted, overall representing 25 chemicals. Secondly, to investigate the toxicological impacts of these chemicals, alone and in mixtures (at equimolar and real-life exposure proportions), the activation of the pregnane X receptor (PXR) on human hepatic cells was used as an endpoint.

The hPXR (NR1I2) is a target for the environmental endocrine disruptor. It belongs to the NR superfamily which evolved from a ligand-activated ancestral receptor into 48 different members in humans. Its function is to sense the presence of foreign toxic substances and upregulate the expression of proteins involved in their clearance from the body (Kliewer, 2003). However, its importance extends far from the regulation of hepatic enzymes and transporters, involved in xenobiotic protection (Reschly and Krasowski, 2006). The role of hPXR is now well established in ameliorating liver injury, increasing cholesterol metabolism and numerous other functions (di Masi *et al.*, 2009). Its activation has also been implicated in a number of clinically adverse drug-drug interactions and may be deleterious in some tissues, as demonstrated by decreased delivery of drugs in the brain (Lombardo *et al.*, 2008), exacerbation of triglycerides and phospholipids accumulation in liver, and in patients with "metabolic syndrome" (di Masi *et al.*, 2009). In cancer growth and carcinogenesis, PXR can acts as an oncogene by inducing cell division (di Masi *et al.*, 2009) protecting against apoptosis (Zucchini *et al.*, 2005) and lowering the efficacy of drug therapy (Mani *et al.*, 2013). This receptor appears an ideal candidate for chemical interactions studies, because it's large, spherical, binding domain cavity (volume can expand from 1300 to 1600 $Å^3$) allows it to accommodate wide ranges of unrelated chemicals with low affinity (Ekins *et al.*, 2007).

Our aim was to evaluate the transactivation potency of the seven pesticide mixtures in a hepatoma cell line expressing the hPXR and the expression of its target gene, CYP3A4 in human hepatocytes. Given the specific mode of action studied, i.e., hPXR activation, we then estimated the effects of the most potent hPXR-activating mixtures and their individual components, by applying the CA model (Bliss, 1939).

## MATERIALS AND METHODS

*Chemicals.* All pesticides (purity > 98%) were purchased from Sigma-Aldrich (Saint-Quentin Falavier, France). Dimethylsulfoxide (DMSO, purity > 99.8%) was also obtained from Sigma-Aldrich. All chemicals were prepared in DMSO and stored at −20°C. Unless stated otherwise, all remaining solutions or chemicals were purchased from Sigma-Aldrich. Table 1 provides a list of the pesticides and their relative proportions in the different mixtures used in the study.

*Cell culture.* The HepG2/hPXR-luciferase cell line was cultured as previously described (Lemaire *et al.*, 2004). These cells were routinely cultured in DMEM (PAA, France) supplemented with 10% fetal bovine serum (FBS) (PAA). The cells were kept in a humidified incubator at 37°C with 5% $CO_2$.

*hPXR activation assay.* HepG2/hPXR-luciferase cells were seeded in sterile white 96-well plates and grown until 70% confluent. The medium was then removed and replaced with 0.1 ml of media: phenol red free DMEM containing 10% charcoal stripped and lipid depleted FBS (PAA). After 24 h, the media was removed and replaced with 0.1 ml assay media containing the appropriate concentration of test compound or mixture. Rifampicin (RIF) 10μM was used as positive control and the DMSO concentration was kept to 0.25% (vol/vol) in all treatments. To determine the reporter gene activity, the luciferase assays were performed using the BriteLitePLus kit (Perkinelmer, France). All compounds and mixtures were tested in at least three independent experiments and were analyzed in duplicate or triplicate. The luminescence signal (Luc) for the tested pesticides and mixtures were normalized to the signal induced by RIF (10μM). NT corresponds to non-treated cells.

$$\text{Normalized response of sample} = \frac{\left(\text{Luc}_{\text{Sample}} - \overline{\text{Luc}}_{\text{NT}}\right) - \left(\overline{\text{Luc}}_{\text{DMSO}} - \overline{\text{Luc}}_{\text{NT}}\right)}{\left(\overline{\text{Luc}}_{\text{RIF}} - \overline{\text{Luc}}_{\text{NT}}\right) - \left(\overline{\text{Luc}}_{\text{DMSO}} - \overline{\text{Luc}}_{\text{NT}}\right)}.$$

*Cell viability assay.* The cytotoxicity of the individual pesticides and pesticide mixtures was evaluated using the xCELLigence real-time cell analyzer in 96-well plates. This device measures the relative cellular impedance changes over time (24 h) at the cell surface to determine physiological changes. All compounds were tested in two independent experiments in triplicate. The formula for the normalized cell index (NCI) calculation, as well as the principles of data assessments have been described previously (Atienza *et al.*, 2005).

*Nonlinear regression modeling.* Statistical dose response analysis was conducted on the whole data set for each individual pesticide and the pesticide mixtures unless a decrease in luciferase response occurred at the highest concentration tested. The bootstrapping technique was used (Efron and Tibshirani, 1986). The normalized original data set obtained (three independent experiments in duplicate) was resampled, with replacements, to create 600 data sets. To analyze the dose response regression for this whole data set, we used the new parameterization of the

**TABLE 1.** Characteristics of the Pesticides and the Percentage Composition of the Pesticides in Each Mixture

| M 1 | M 2 | M 3 | M 4 | M 5 | M 6 | M 7 |
|---|---|---|---|---|---|---|
| **Apples, Pears** | **Carrots, turnips, celeriac** | **Table grapes** | **Vegetables, fruits,** | **Fish products** | **Potatoes, onions** | **Citrus fruits, bananas** |
| *Propargite*[a] *42%* | **Chlorfenvinphos 61%** | Fenhexamid[a] 52% | *Procymidone*[a,b] *42%* | **DDT**[*,b] **95%** | Maleic hydrazide 74% | Imazalil[a,b] 97% |
| *Diphenylamine 40%* | **Ethion**[b] **25%** | Pyrimethanil 31% | Iprodione[b] 33% | **Dieldrin**[*,b] **5%** | Chlorpropham[a,b] 26% | **Methidathion**[b] **3%** |
| **Phosalone**[b] **13%** | Linuron[a,b] 14% | *Fenitrothion*[a, b] *9%* | Cyprodinil[a] 15% | | | |
| Captan[b] 3% | | Triadimenol 6% | Fludioxonil[a] 9% | | | |
| **Tolylfluanid**[a] **2%** | | Quinoxyfen[a] 2% | Cyhalothrin[a, b] 1% | | | |
| | | Penconazole 1% | | | | |

*Note.* Pesticides in bold were banned at the date of the Crepet *et al.* study. Pesticides in italic are now banned. Underlined pesticides are authorized in the European Union.
[a]Pesticides present in the Orton study.
[b]Pesticides present in the Kojima study.
[*]Pesticides classified as persistent organic pollutant.

five-parameter logistic (5PL) (Liao and Liu, 2009) function Equation (1), which takes the curve asymmetry into consideration (Gottschalk and Dunn, 2005) to overcome some drawbacks of the four-parameter logistic (4PL) functions and improve mixture assessment (Dawson *et al.*, 2012). This new formulation, given in the following equation, also preserves the practical useful parameters of the 4PL by identifying the $EC_{50}$ as the "c" parameter.

$$y = f(x;p) = f(x;a,d,c,b,g) = (d+(a-d))/(1+(2^{(1/g)}-1)*(x/c)^b)^g \quad (1)$$

where $x$ = concentration, the model parameters were as follows: $d$, the minimal; $a$, the asymptotic maximal effect; $c$, the $EC_{50}$; $b$, the shape parameter; and $g$, the curve asymmetry. For each bootstrap, coefficient calculations were made using an iterative (algorithm of Levenberg-Marquardt) procedure. Concentration effects were determined for the 600 bootstrap data sets by the functional inverse Equation (2):

$$x = c*((-1+((a-d)/(EC_x-d))^{(1/g)})/(-1+2^{(1/g)}))^{(1/b)} \quad (2)$$

We calculated, $y = f(x;p)$, for $x$ from 0 to 99, with a step of 0.033 for the 600 nonlinear regression (NLR) models. A scatter plot of the normalized luciferase activity versus concentration was then constructed by tracing the mean of $y$, the 2.5th and the 97.5th percentiles for each $x$. A Lowess fitting method (STATISTICA) was used to fit the pairs of points with the stiffness parameter set to zero.

For the pilot study, which was designed to choose three mixtures out of the seven, dose responses were tested at five concentrations (two experiments in triplicate). Data were visualized using a Box Plot. To select the three mixtures with the greatest potency at 10μM, a one-way ANOVA followed by Tukey's HSD *post hoc* test was used to provide homogeneous groups for the means. Means within the same subset were not significantly different from each other at a probability of 0.05.

*Calculation of the predicted effect of the mixtures.* The CA model was used to model the theoretical concentration relationship for the three mixtures of pesticides both at equimolar and real-life exposure proportions. As the proportion of mixture ($p_i$) components was known and constant, the calculation of the predicted effect $ECx_{mix}$ was calculated using Equation (3) (Faust *et al.*, 2003):

$$ECx_{mix} = \left(\sum_{p_i=1}^{n} \frac{p_i}{F_i^{-1}(x)}\right)^{-1} \quad (3)$$

To construct the prediction curve and maintain the variability obtained after bootstrapping, the predictive CA model was calculated for the 600 NLR from each chemical dose response. The estimated concentration/response curve was then constructed by tracing (scatter plot): mean ± 2.5th and 97.5th percentiles. Differences between the predicted and observed effects at doses were deemed statistically significant when the percentile confidence belts did not overlap.

*Determination of concentrations inducing 50 and 10% of the normalized effects of RIF at 10μM.* All data were normalized to the hPXR agonist, RIF (fixed to 1). The effective concentrations producing 50 or 10% (i.e., 0.5 and 0.1 on the graph) of the normalized effects of RIF were noted as $EC_{vsrif0.5}$ and $EC_{vsrif0.1}$. These values were calculated from the inverted nonlinear curve fitting of the whole data set after bootstrapping and expressed as: mean concentration ± 2.5th and 97.5th percentiles. The pesticides were classified, on the basis of the concentration that produced an effect of 0.5 (relative to RIF), as strong ($EC_{vsrif0.5} < 5\mu M$), medium ($5\mu M < EC_{vsrif0.5} < 25\mu M$) and weak ($EC_{vsrif0.5} > 25\mu M$). This classification more effectively takes into account the relative potency of hPXR

agonists, than $EC_{50}$, which largely depends on the efficacy of the molecule.

*Statistical analyses.* All statistical analyses and graphical representations were performed using the STATISTICA software (version 10.0 or 12.0, Statsoft, France).

*Human hepatocyte cultures and treatments.* Cryopreserved human hepatocytes were cultivated as previously described (de Sousa *et al.*, 1996; Lemaire *et al.*, 2004). Hepatocytes were treated for 3 days with 10μM (medium without serum) of the pesticide mixtures alongside RIF as a positive control and DMSO as a negative control.

*Western blot analysis.* After treatment, cells were lysed and proteins were loaded onto a 12% SDS/PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore, France). The membrane was incubated with anti-CYP3A4 primary antibodies (Oxford, UK). After incubation with horseradish-peroxidase-conjugated secondary antibodies (Promega, France), immunodetection was performed using the luminescence detection kit (Millipore).

## RESULTS

A pilot study was designed firstly to assess the efficacy of the seven mixtures (Table 1) for hPXR transactivation. HepG2/hPXR cells were exposed to the cocktails, at five concentrations (1, 3, 10, 30, and 100μM) with the pesticides at equimolar proportions. Figure 1 shows the concentration responses for the activation of hPXR by the seven mixtures (fold activation over DMSO), as well as the dynamic monitoring of changes in the cell population, as measured by real-time cellular impedance and analyzed using the NCI. For most of the mixtures, except M1, the luciferase activity increased with treatment up to 30μM. These results were consistent with the expression of the prototypical hPXR target gene, CYP3A4, in human hepatocyte primary cultures, treated with the mixtures at 10μM (Fig. 1H, insert). The decrease in the luciferase activity observed at the high concentrations of the mixtures can be explained for most of them, by a cytotoxic effect. The most toxic mixture was M1 with a decrease in NCI beginning at 10μM as soon as 5 h after treatment. A clear toxic effect was observed for M3 and M5 at 100μM, with a continuous decrease in the NCI after ±6–24 h. For the M4 mixture, the NCI decreased, from 6 to ± 16 h at 30 and 100μM, then cells tended to recover as shown by an increase in the NCI value. For M2 and M7, a slight decrease (20%) in the NCI occurred only at high concentrations. Only M6 appeared to be devoid of any toxicity over the whole range of the concentrations tested.

The mixtures that caused the highest activation of hPXR were then classified by a one-way ANOVA followed by a *post hoc* analysis which divided the luciferase activity induced by the mixtures (10μM), into four homogeneous groups (Fig. 1H). The two groups with the highest potency were formed by M2 (group no. 1) and M3, M4 (group no. 2), which were accordingly retained for further studies. The rank order of the mixtures for their ability to activate hPXR was as follows: M2 > M4/M3 > M5/M1 > M7/M6.

*Dose-Response Modeling of hPXR Activation by the Individual Components of Mixtures 2, 3, and 4*

As an hPXR agonist, RIF [maximal response (efficacy): 10μM, $EC_{50}$ = 1.8μM] (Lemaire *et al.*, 2004) was used as a positive control and its $EC_{50}$ was used as the reference value to classify the pesti-

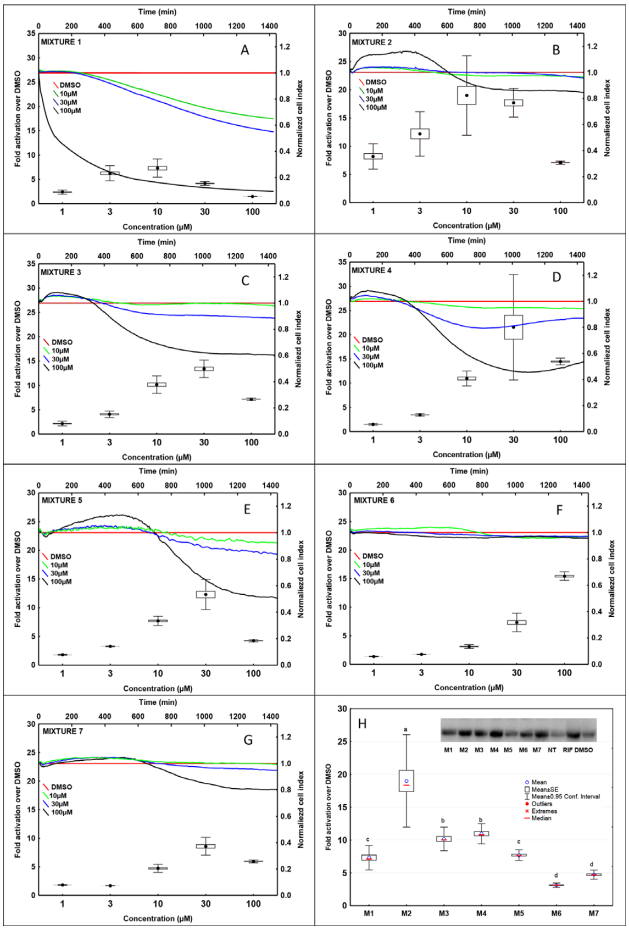


**FIG. 1.** Results of the pilot assay. The dot plot (A–G) represents the multi-concentration (10–100μM) time-dependent cellular response curves of the mixtures (1–7) over a 24-h period (upper abscissa, right ordinate). Increasing the concentration led to a decrease in cell index which reflects mainly the dynamic evolution of the cell population. The box plot represents the fold activation of luciferase activity under hPXR control, over the DMSO, (H) comparison of the luciferase activity over the DMSO control for the whole set of the mixtures at 10μM [mean, median, ±SE (whiskers); 0.95 confidence interval (lower abscissa, left ordinate)], ANOVA indicated five homogenous groups after Tukey's HSD *post hoc* test $p \leq 0.05$. The insert represents the expression of CYP3A4 in human hepatocytes. Human cryopreserved hepatocytes were treated with 10μM of each mixture at equimolar proportions for 3 days (medium was renewed every 24 h). Cells were then subjected to Western blotting.

cides. The three selected mixtures consisted of a total of 14 pesticides (Table 2). The dose-response curves of hPXR activation, represented by the Lowess smoothing of data (pairs of mean effect/concentration, see Materials and Methods section) were perfectly fitted to the mean of the experimental data set (Fig. 2). There was a great discrepancy in terms of efficacy and potency for the various pesticides, with some pesticides having higher efficacy than RIF. When compared with this agonist (efficacy set to 1), 7 pesticides of the 14 which constituted the three mixtures, exhibited higher efficacy (>1.2) and for two of them this efficacy was >1.6 (Fig. 2). Goodness of fit of nonlinear regression, modeled after bootstrapping, was tested for pesticides with large variation (e.g., triadimenol) or when data were eliminated due to a lower luciferase activity at high concentration (e.g., chlorfenvinfos), for the 600 nonlinear regressions. The mean of least squares deviation (LSD, mean squared error) was less than ±0.03

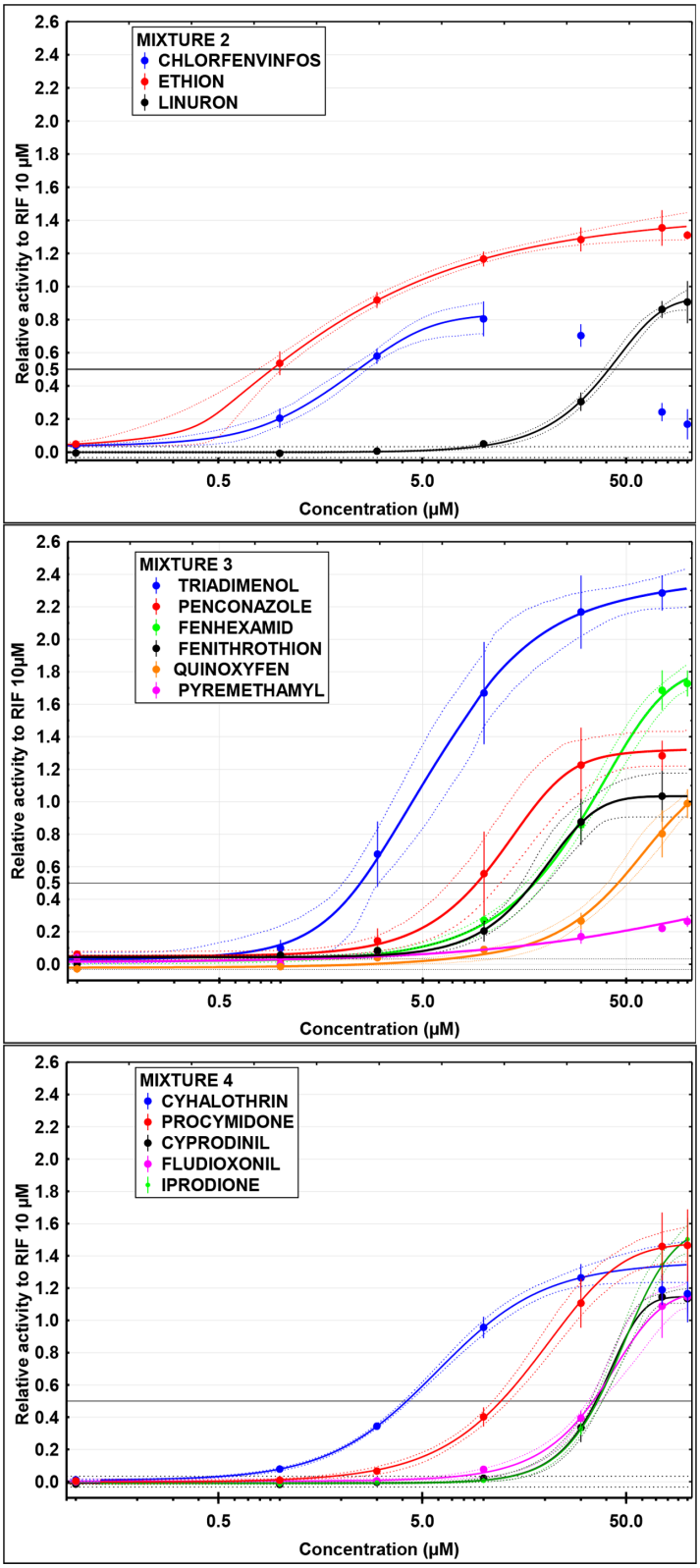


FIG. 2. Scatter plot of the concentration-response analysis for the individual pesticides from mixtures 2, 3, and 4. Solid line is a Lowess fitting of the mean response estimated from the 600 nonlinear regressions. Dashed lines are the 2.5th and 97.5th percentiles. Data shown are mean ± SD of three independent experiments. The horizontal black dotted lines are the 95% CI of the mean of DMSO treated cells.

(0.04 for the 97.5th percentile) and the correlation coefficient was higher than ±0.98 (0.96 for the 2.5th percentile).

Concerning mixture 2, ethion showed a greater potency and efficacy when compared with RIF (≈2-fold), followed closely by chlorfenvinfos (≈equipotent), then by linuron which was much less active (Fig. 2A; Table 2). Surprisingly, for the majority of the pesticides, the decrease in luciferase activity could be related to their toxic effects at high concentrations; however, this was not the case for chlorfenvinfos, when the luciferase activity decreased from 10 to 100μM, without clear evidence of cytotoxicity, as revealed by the viability test used (data not shown).

Regarding the pesticides of the mixture M3 (Fig. 2B; Table 2), despite a wide range of chemical structures, most were capable of activating the hPXR. Comparisons of their $EC_{vsrif0.5}$ only showed triadimenol as a strong activator of hPXR (Table 2).

Pesticides composing the M4 mixture could be organized into three separate clusters, with significantly different hPXR potency. Cyhalothrin was shown to be the most potent hPXR activator, behaving like a strong hPXR agonist, followed by procymidone, and finally fludiodoxonyl, cyprodinil, and iprodione (Fig. 2C; Table 2). Surprisingly, a pronounced toxic effect did not necessarily lead to a frank decay in luciferase activity, as was the case, e.g., for fludioxonil (data not shown).

*Experimental and Predicted Effects of the Mixtures at Both Equimolar Concentrations and Real-Life Exposure Proportions*

*Mixture 2.* Of the three chemicals which composed M2 (mostly found in fish), two were strong hPXR activators, chlorfenvinfos, and ethion. Chlorfenvinfos was shown to decrease luciferase activity without affecting viability (real-time impedance analysis). Its high proportion in the mixture did not permit modeling after 30μM at equimolar proportions or 10μM at real-life exposure proportions. The CA model yielded accurate predictions both at equimolar concentrations or real-life exposure proportions (Figs. 3A and 3B; Table 3).

*Mixture 3.* This mixture was composed of six pesticides (five fungicides and one organophosphate) and was mostly found in grapes. For this mixture (Figs. 3C and 3D; Table 3), a correct prediction of the CA model was obtained up to ±9μM at equimolar proportions, as shown by the overlapping curves below this concentration and almost similar $EC_{50}$ values. Above this concentration, a less than additive effect was observed. At real-life exposure proportions, nonoverlapping curves and a significant but slightly (<2-fold) different $EC_{vsrif0.5}$ suggested a less than additive effect on hPXR activation.

*Mixture 4.* This mixture consisted of five pesticides which were mostly found in vegetables and fruits (four fungicides, one pyrethroid). This mixture was composed mainly of medium and weak hPXR activators as compared with M2 and M3 (Figs. 3E and 3F; Table 3). Only the observed effects for equimolar proportion mixture fell short of the predictions. Predicted curves only showed a similar slope to that of the experimental one (Figs. 3E and 3F). Observed effects were 1.5-fold lower than predicted effects ($EC_{vsrif0.5}$) and in the same range at $EC_{vsrif0.1}$. Finally at equimolar proportions, a greater than additive effect was observed with much lower values for the experimental $EC_{vsrif0.5}$ than the theoretical ones (Table 3). Surprisingly, at real-life ratios, a less than additive effect was obtained with an experimental data curve shift to the left of the modeled one.

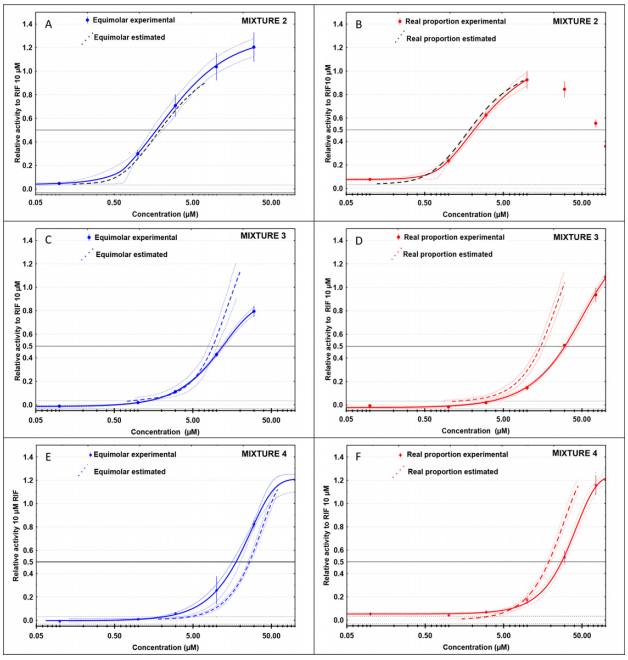


**FIG. 3.** Predicted and observed hPXR activation by the mixtures at equimolar proportions and real-life exposure proportions. The scatter plot represents the mean response from the 600 nonlinear regressions. Dotted lines are the 2.5th and 97.5th percentiles. Data shown are the mean ± SD. The predicted effect curve (dotted line) was calculated from the whole set of nonlinear regression after bootstrapping, using the CA model. Dashed lines are the 2.5th and 97.5th percentiles for the observed and predicted effects. The observed mixture effect is the mean ± SD of three independent experiments. The horizontal black dashed lines are the 95% CI of the mean of the DMSO-treated cells. Due to the perfect overlap of the predicted and observed scatter plot for mixture 2, the percentile was not represented for the predicted curve.

## DISCUSSION

With much alarming data concerning the chemical contamination of food (Guillette and Iguchi, 2012) and given the lack of that on human bio-monitoring, this research addresses and confirms the concerns of the European and U.S. agencies, that "even at low levels of exposure, complex mixtures of pollutants can have significant effects on health."

Numerous pesticides are known to be agonists and/or antagonists for most of the nuclear receptors like estrogen, androgen, thyroid hormone, constitutive androstane, peroxisome proliferator-activated receptor, and the PXRs, as well as the aryl hydrocarbon receptor. Some of these chemicals also have dual actions and interact with more than one NR. However, when compared with endogenous hormones, the agonist efficacy of most pesticides is considered to be low. Nevertheless, what is well known now is that all of these pesticides can act together to activate some of these nuclear receptors, and despite observed deviations, the CA model is the suitable model to predict the effects of mixtures (Kortenkamp *et al.*, 2012).

hPXR drives the cellular machinery to handle and eliminate from the body toxic compounds either foreign or derived from endogenous metabolism (Hernandez *et al.*, 2009). However, research has revealed a "Mr Hyde nature" of the PXR (Biswas *et al.*, 2009), and that its activation can also impact health and disease. Despite the fact that it is activated by a very large number of environmental, occupational, and dietary chemicals, nobody to our knowledge has yet performed investigations on the potential impact of pesticide mixtures on this receptor. Numer-

**TABLE 2.** Characteristics of the Concentration-Response Relationships of Each Pesticide from the Different Mixtures

| Chemical | $EC_{vsrif0.5}$ [a,b] | $EC_{vsrif0.1}$ [a,b] | $EC_{50}$ (μM) | Group | REP |
|---|---|---|---|---|---|
| Mixture 2: | | | | | |
| Ethion | 0.91 (0.87, 0.96) | 0.31 (0.15, 0.51) | 1.7 (1.5, 1.9) | S | 1.98 |
| Chlorfenvinfos[c] | 2.4 (2.1, 2.7) | 0.55 (0.36, 0.76) | 2.2 (2.1, 2.3) | S | 0.75 |
| Linuron | 41.1 (38.2, 44.6) | 16.2 (14.1, 18.9) | 39.3 (35.0, 45.5) | W | 0.044 |
| Mixture 3: | | | | | |
| Triadimenol[c] | 2.55 (2.02, 3.04) | 0.95 (0.47, 1.87) | 6.0 (5.8, 6.1) | S | 0.71 |
| Penconazole[c] | 9.40 (6.8, 12. 1) | 2.5 (1.6, 3.7) | 13.03 (12.6, 13.5) | M | 0.19 |
| Fenitrothion[c] | 17.74 (14.8, 20.6) | 5.95 (4.24, 7.46) | 18.83 (18.6, 19.1) | M | 0.10 |
| Fenhexamid | 17.78 (16.0, 20.0 | 4.73 (3.87 -6.11) | 31.5 (31.2, 31.7) | M | 0.10 |
| Quinoxyfen | 45.83 (40.3, 51.2) | 14.85 (10.73, 19.04) | 56.1 (54.9, 57.26) | W | 0.022 |
| Pyremethanil | 189 (extrapolated[d]) | 22.41 | NC | W | NC |
| Mixture 4: | | | | | |
| Cyhalothrin[c] | 4.2 (4.04, 4.35) | 1.2 (1.1, 1.3) | 6.05 (5.18, 7.88) | S | 0.43 |
| Procymidone | 12.2 (11.1, 13.3) | 3.7 (3.1, 4.6) | 18.6 (15.2, 22.8) | M | 0.15 |
| Fludioxonil | 34.6 (32.5, 37.5) | 13.9 (11.3, 17.2) | 40.7 (35.0, 56.6) | W | 0.052 |
| Cyprodinyl | 35.6 (33.3, 38.2) | 19.2 (16.7, 21.9) | 37.9 (35.1, 41.3) | W | 0.051 |
| Iprodione | 36.3 (34.7, 38.24) | 19.3 (17.0, 21.1) | 48.4 (41.3, 68.5) | W | 0.050 |

*Note*. The concentration effect was estimated using regression models after bootstrapping.
[a]Concentrations that induce an effect of 0.5 or 0.1 with respect to RIF.
[b]CIs as defined by the 2.5th and 97.5th percentiles. Groups: pesticides were classified according to their concentrations that induce an effect of 0.5: S: strong, M: medium, W: weak inducer.
[c]Decrease in the luciferase activity at high concentrations. REP: relative potency with respect to RIF.
[d]Extrapolated from the Figure 2.

**TABLE 3.** Characteristics of the Observed and Predicted Effects of the Mixtures

| | $EC_{vsrif0.5}$ [a,b] (μM) | $EC_{vsrif0.1}$ [a,b] (μM) |
|---|---|---|
| Mixture 2: | | |
| Observed | | |
| Equimolar | 1.74 (1.5, 2.1) | 0.42 (0.24, 0.73) |
| Real prop | 2.11 (1.9, 2.3) | 0.52 (0.30, 0.80) |
| Predicted by CA | | |
| Equimolar | 1.91 (1.35, 2.3) | 0.42 (0.27, 0.75) |
| Real prop | 1.85 (1.43, 2.03) | 0.47 (0.30, 0.68) |
| Mixture 3: | | |
| Observed | | |
| Equimolar | 12.1 (11.6, 12.6) | 2.8 (2.67, 2.85) |
| Real prop | 30.4 (29.2, 32.5) | 7.2 (6.4, 8.0) |
| Predicted by CA | | |
| Equimolar | 9.0 (7.9, 10.2) | 2.96 (1.95, 3.95) |
| Real prop | 16.25 (14.7, 18.1) | 4.8 (3.57, 5.8) |
| Mixture 4: | | |
| Observed | | |
| Equimolar | 17.6 (15.8, 20.2) | 5.04 (4.0, 7.3) |
| Real prop | 27.6 (25.8, 29.3) | 6.43 (5.2, 8.4) |
| Predicted by CA | | |
| Equimolar | 26.3 (25.05, 27.3) | 9.41 (8.57, 10.5) |
| Real prop | 18.85 (17.7, 19.9) | 6.52 (5.76, 7.57) |

*Note*. The concentration effect was estimated using regression models after bootstrapping.
[a]Concentrations that induce an effect of 0.5 or 0.1 with respect to RIF.
[b]CIs as defined by the 2.5th and 97.5th percentiles.

ous studies have shown that pesticides could act as PXR activators. Many of them have already been described as full or partial hPXR agonists, both on stably- (Lemaire *et al.*, 2004) or transiently transfected cell lines (Kojima *et al.*, 2011), the HepaRG cell line (Nawaz *et al.*, 2014) and normal human or animal hepatocytes (Coumoul *et al.*, 2002; Lemaire *et al.*, 2004). However, in these studies, pesticides were selected on the basis either of their presence in the environment or of their known or supposed toxic effect, but never on human exposure data. In contrast to the already published studies, our work is not only based on pesticide mixtures or on individual chemicals, but also on those to which the French general population is most frequently exposed to via their diet. It should be pointed out that the French population is exposed to other chemicals, belonging to different chemical families, and possessing different modes of action. Furthermore, numerous pesticides that were banned from the EEC can still be found in various sources and were thus present in our test mixtures. At the date of the study by Crepet *et al.* (2013),

seven active substances (phosalone, tolylfluanid, chlorfenvinfos, ethion, DDT, dieldrin, and methidation) were banned or submitted with limits for their use. This list has grown with the addition of propargite, diphenylamine, fenitrothion, and procymidone. Although the exposure to nonauthorized pesticides would thus be expected to decrease, we should plan for their continued presence in the French diet due to their persistence in the environment (i.e., DDT in fish) or presence in food commodities imported from outside the European Union. Due to similar agricultural practices, these pesticides can also be found in numerous countries: for instance, 14 and 27% of grapes, in the United States, contain fludioxonyl and cyprodinyl, respectively (Orton *et al.*, 2011; U.S. Environmental Protection Agency, 2011).

Our results on hPXR activation, confirm the current experimental data for most of the individual pesticides common to our study, with only a few discrepancies. In contrast to the study of Kojima demonstrating that iprodione and linuron could be classified as positive hPXR agonists (Kojima *et al.*, 2011), our data provided evidence of a lower potency of these chemicals for hPXR activation. This extensive study investigated 200 pesticides, only 13 of which are among the 25 pesticides (50%) to which the French population is exposed to through their diet; and for the three mixtures with the highest agonist action toward hPXR, this proportion decreased to 40% (6/14). For the other studies, this proportion varied from 1/29 to 2/15 (Lemaire *et al.*, 2004, 2006). This point highlights the fact that previous studies do not focus on the actual pesticides the French population are exposed to. There is also a discrepancy between the lists of pesticides in the studies of Crepet *et al.* (2013) and Orton *et al.* (2011) based on exposure. Indeed, they tested for the antiandrogenic effect of 37 pesticides which were selected on the basis of exposure data in the European Union. Thirteen of these pesticides were also found in the seven mixtures defined by Crepet *et al.* (2013) and eight were present in the three mixtures that we extensively studied (8/14—60%), with a large prevalence of fungicides in both studies. These data highlight the need for more detailed characterization of data on human exposure, to focus investigations on pesticides and pertinent mixtures to which the general population are exposed to in real-life.

Moreover, as demonstrated by Crepet *et al.* (2013), pesticides are not found in equal proportions in a mixture. Therefore, in order to mimic human exposure, we studied pesticide mixtures both at equimolar concentrations or real-life exposure proportions. For the three mixtures we have studied, only M4, led to a shift from a less than additive effect when in real-life proportion to a greater than additive effect when in equimolar proportions.

Overall, when considering a mixture with potent hPXR agonist activity, i.e., M2, the CA model perfectly fitted experimental data, and the pesticide concentration that induced 0.5 of the full efficacy of RIF, was obtained as low as 0.6μM for the three chemicals. This pesticide mixture is mostly found in fish foods, which are known to be contaminated by many POPs considered as EDCs (Hotchkiss *et al.*, 2008). Acting together and via multiple pathways and receptor-based mechanisms, all of these chemicals can affect human health.

Despite the small variations that we have observed and due to the conservative predictions from CA, this is a suitable model for estimating the effect of mixtures on hPXR activation and its impact on human health (Kortenkamp *et al.*, 2012). For the risk assessment, additional *in vivo* studies are needed to confirm that a combination of potent pesticides which activate hPXR may act additively and lead to a chronic activation of hPXR. However, as PXR shows the highest degree of cross-species variation in the ligand-binding domain of the entire vertebrate NR superfamily (Ekins *et al.*, 2008; Reschly and Krasowski, 2006), animal studies using human bio-monitoring data will surely fail to predict the effect of an individual chemical (Shukla *et al.*, 2011) or mixture unless a humanized animal model (Gonzalez, 2007) and physiologically based pharmacokinetic (PBPK) model is used (Tan *et al.*, 2011). Recently the development of a mathematical model has made it possible to predict the profile of CYP3A4 induction in humans from data obtained from *in vitro* reporter gene assays, provided that some PBPK information on molecules are known (Kozawa *et al.*, 2009). Another recent study has shown that doses as low as 20 mg/day of RIF, which is much lower than the therapeutic dose (400–600 mg/day), taken by healthy subjects for 14 days can significantly induce the expression of CYP3A4, through hPXR (di Masi *et al.*, 2009; Kanebratt *et al.*, 2008). Therefore, it is likely that low doses of potent pesticides in mixtures can have the same effect as long as the contamination occurs chronically even at the ADI. There is now evidence that chronic activation of PXR can have many implications considering that the detoxification proteins induced are responsible for the metabolism, deactivation and transport of bile acids, thyroid and steroid hormones, numerous environmental chemicals, and several drugs (Mani *et al.*, 2013). As the two most targeted genes of hPXR are CYP3A4 and MDR1, hPXR activation can in particular decrease the efficacy of drugs used for anticancer therapy, which has led to pharmaceutical companies screening for PXR antagonists. Hence, it is increasingly clear that food contaminants such as pesticides can act together and with other xenobiotics on the hPXR, and thereby disrupt cellular homeostasis and therapeutic behavior. *In vitro* activation of nuclear receptors over the next few years will become a high throughput tool and a simple means of assessing the hPXR agonist potency of pesticides alone and in a mixture.

In conclusion, the present study demonstrates that mixtures of pesticides found in the French diet mostly act additively as hPXR agonists. Such activation may represent a risk factor contributing to endocrine system disorders in humans. Such mixtures of pesticides acting together with an additive effect, could contribute to undesirable effects in humans and impact health, exacerbate pathology and modify the therapeutic properties of drugs through hPXR activation. There is undeniably an increasing need to address the potential risks of combined exposure to pesticide cocktails in the diet and to update the pesticide legislation which currently only considers active substances taken individually. The mechanistic cause for the deviation from CA observed with some hPXR agonist mixtures is unknown Because any such deviation was only minor, the CA model is recommended for risk assessment as it typically more conservative than the alternative, independent action model.

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